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14. ABSTRACT

While adoptive immunotherapy holds promise as a treatment for cancer and infectious diseases, development has been impeded by the lack of reproducible methods for generating therapeutic numbers of antigen-specific CD8+ CTL. As a result, there are only limited reports of expansion of antigen-specific CTL to levels required for clinical therapy. Therefore, our groups has previously developed artificial Antigen-Presenting Cells (aAPC), made by coupling soluble HLA-Ig and anti-CD28 to beads. These aAPC have successfully been used to induce and expand CTL specific for CMV or melanoma. For the current study we have proposed to used and further developed those aAPC for the generation of prostate cancer specific CTL. Our preliminary data demonstrate that aAPC loaded with the prostate cancer specific antigen EpHA2 have been used to generate functional active prostate cancer-specific CTL from peripheral blood healthy donors.

15. SUBJECT TERMS
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Table of Contents

INTRODUCTION
While adoptive immunotherapy holds promise as a treatment for cancer, development of adoptive immunotherapy has been impeded by the lack of a reproducible and economically viable method for generating therapeutic numbers of antigen-specific CTL. Therefore, we are studying use of HLA A2-Ig based aAPC for induction and expansion of prostate specific CTL with the goal of replacing the use of autologous DC for adoptive immunotherapy for prostate cancer. More specifically, we will demonstrate functional efficacy of an "off the shelf" HLA-Ig based artificial Antigen Presenting Cells (aAPC) for inducing and expanding anti-EphA2(58) or $PSMA_{27}$ prostate-specific CTL. The specific aims are to *1) optimize aAPC structure and duration of stimulation, and 2) analyze the in vivo function of aAPC-induced CTL.*
These studies will serve as precursor ones for induction and expansion of prostate specific CTL from patients with disease for initiation of adoptive immunotherapy clinical studies as an adjuvant therapy post surgery in the setting of minimal residual disease.

BODY

Adoptive immunotherapy for prostate cancer has been limited by the use of autologous dendritic cells (DC) for expansion of prostate cancer-specific CTL. Recently, we have shown that HLA-A2-Ig based aAPC can be used to expand model antigen specific CTL. To evaluate this approach for use in prostate cancer immunotherapy we proposed to study initially aAPC based *in vitro* expansion of prostate cancer specific CTL using blood from healthy donors and later to evaluate the *in vivo* efficacy of these CTL using a human/SCID mouse model. Over the past award time we have focused on our goals as identified in the statement of work, specifically on optimization of aAPC based T cell stimulation and induction of EphA2 and PSMA specific CTL.

Figure 1: Schematic for aAPC based induction and expansion of induction and expansion of antigen specific CTL
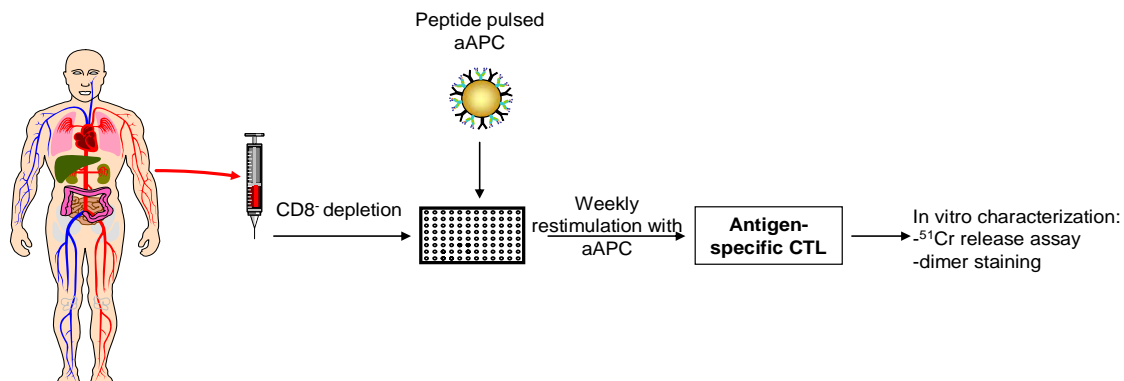
Legend: $CD8^+$ T cells were isolated from peripheral blood of healthy donors using magnetic $CD8^-$ depletion and co-cultured as shown in the schematic for 4-6 weeks. $CD8^+$ T cells were co-cultured with peptide loaded aAPC and harvested once a week. The old beads were removed; T cells were counted and replated and restimulated in 96 well plates with fresh aAPC. After 3 weeks cells were tested as described. Cultures were maintained until the total cell count dropped below $5x10^5$.

We performed aAPC stimulation as described in Figure 1 for multiple donors with PSMA, EphA2 and control peptides like CMV or Mart-1. After several weeks of culture we were able to detect EphA2 specific T cells from 3 donors, whereas no PSMA specific T cells could be generated. At the same time control cultures using Mart-1 or CMV loaded aAPC generated large numbers of highly specific T cells. Figure 2 shows one representative example. The specificity and functionality of the EphA2-specific CTL was confirmed by specific lyses of peptide pulsed target cells in an ^{51}Cr-release assay (Figure 3).

Figure 2: Dimer/tetramer analysis of aAPC induced CTL after 3 weeks of culture
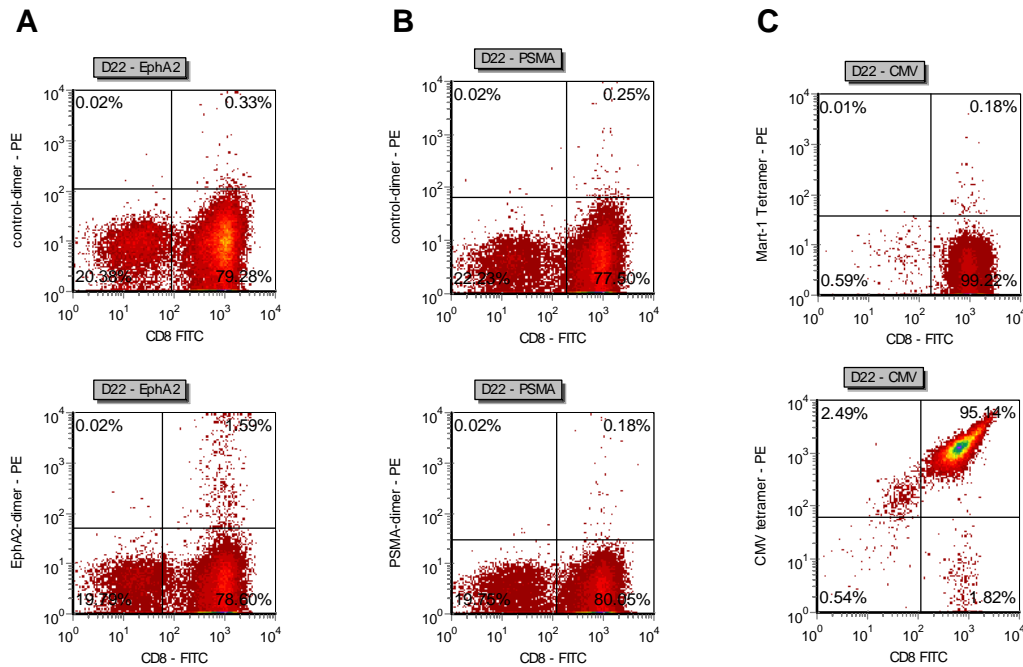

Legend: Figure 2 shows dimer/tetramer staining of T cells which were stimulated for 3 weeks with A) EphA2 loaded aAPC, B) PSMA loaded aAPC and C) CMV loaded aAPC. While the CMV loaded aAPC induced high numbers of antigen specific CTL, aAPC loaded with the prostate cancer specific peptides induce only small numbers using the EphA2 peptide and no specific T cells using the PSMA peptide.

Since the initial approach was not successful for generation of PSMA specific CTL and the generation of EphA2-specific CTL was limited in numbers, we have started to develop second generation aAPC formulations as proposed in the statement of work. B7.1 (CD80) and B7.2 (CD86) on DC are the natural ligands to CD28 on T cells. In addition, it has been reported that the engagement of CD83 on DC with his currently still unknown ligand on T cells can support proliferation and at the same time reduce T cell apoptosis. Our standard aAPC was made by using HLA-A2-Ig in combination with an

antibody specific for CD28 on T cells. We postulated that the natural ligand might have higher affinity or activity and therefore might induce better or stronger costimulation.

Figure 3: Cytotoxic activity of aAPC induced CTL determined by 51Cr-release assay
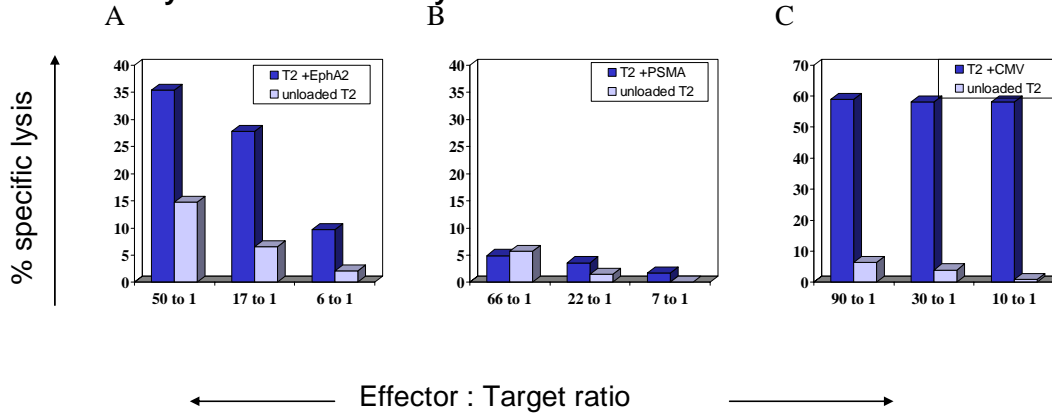
Legend: Figure 3 shows the cytotoxic activity of aAPC induced peptide specific CTL. CD8^{+} T cells were stimulated for 3 weeks with either EphA2 loaded aAPC (A), PSMA loaded aAPC (B) or CMV loaded aAPC (C) and then tested for their cytotoxic activity using a standard ^{51}Cr-release assay. Therefore, total T cells were incubated with either peptide loaded target cells or unloaded target cells as negative control. The given ratios are the ratios from total T cells to target cells.
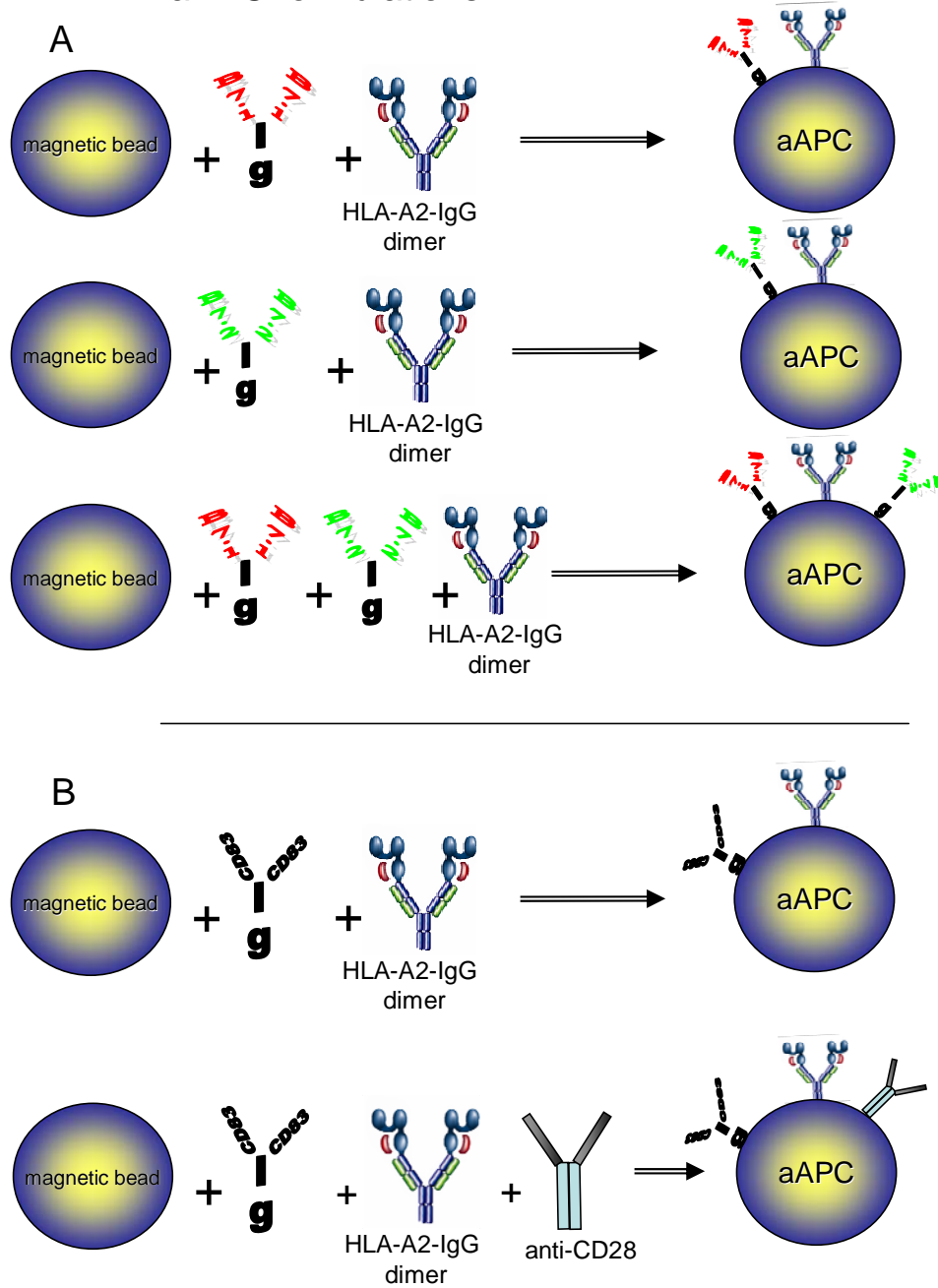
Therefore, we created new aAPC by coating B7.1, B7.2 or B7.1 and B7.2 together with HLA-A2-Ig on magnetic beads. In addition we also generated aAPC by coating magnetic beads with HLA-A2-Ig in combination with CD83-Ig +/- anti-CD28.
Figure 4 shows a schematic of the new created aAPC.

To investigate these new systems we compared the stimulation of the new aAPC with our prototype anti-CD28 based aAPC in either the Mart-1 or in case of the CD83-based aAPC in the CMV system. Both are robust systems which work well for induction of functional antigen-specific CTL as shown in our previous work(1, 2).

We found that in 2 of 3 experiments B7-1 based aAPC generated a higher frequency of antigen-specific CTL than our standard aAPC, figure 5 shows one representative experiment. For one donor we also found a much higher proliferation compared to all other aAPC formulations tested (data not shown).

Figure 4: Schematic of second generation aAPC formulations

A

magnetic bead + g + HLA-A2-IgG dimer ⟹ aAPC

magnetic bead + g + HLA-A2-IgG dimer ⟹ aAPC

magnetic bead + g + g + HLA-A2-IgG dimer ⟹ aAPC

B

magnetic bead + CD83/CD83 g + HLA-A2-IgG dimer ⟹ aAPC

magnetic bead + CD83/CD83 g + HLA-A2-IgG dimer + anti-CD28 ⟹ aAPC

Legend: Figure 4 shows schematically the structure of various formulations of second generation aAPC. 4A shows aAPC based on the use of B7.1- and B7.2-Ig and 4B aAPC based on the use of CD83-Ig either alone or in combination with anti-CD28.

Based on these results we have repeated the experiments to induce prostate cancer specific CTL, comparing our standard anti-CD28 based aAPC with the B7.1-Ig based aAPC (Figure 6). We found that in the first 2 experiments that the use of B7.1-Ig based aAPC did not improve the outcome of the resulting T cell product. While the FACS analysis using antigen-specific dimer staining showed the induction of EphA2-specific CTL at comparable levels to our standard aAPC (Figure 6A), further functional analysis showed high non-specific killing for CTL that were induced with B7.1-Ig based aAPC. In contrast CTL which were induced with our regular anti-CD28 aAPC showed only minimal non-specific killing (Figure 6B). This could be due to the high amount of non-EphA2-spepcific cells contaminating the culture. Therefore, further experiments, which are currently ongoing, are necessary to analyze the capacity of the B7.1-Ig based aAPC in more detail. This is specifically important these aAPC seemed to be more potent in the well established Mart-1 system.

Figure 5: Tetramer analysis of 2nd generation B7-Ig based aAPC induced CMV specific CTL
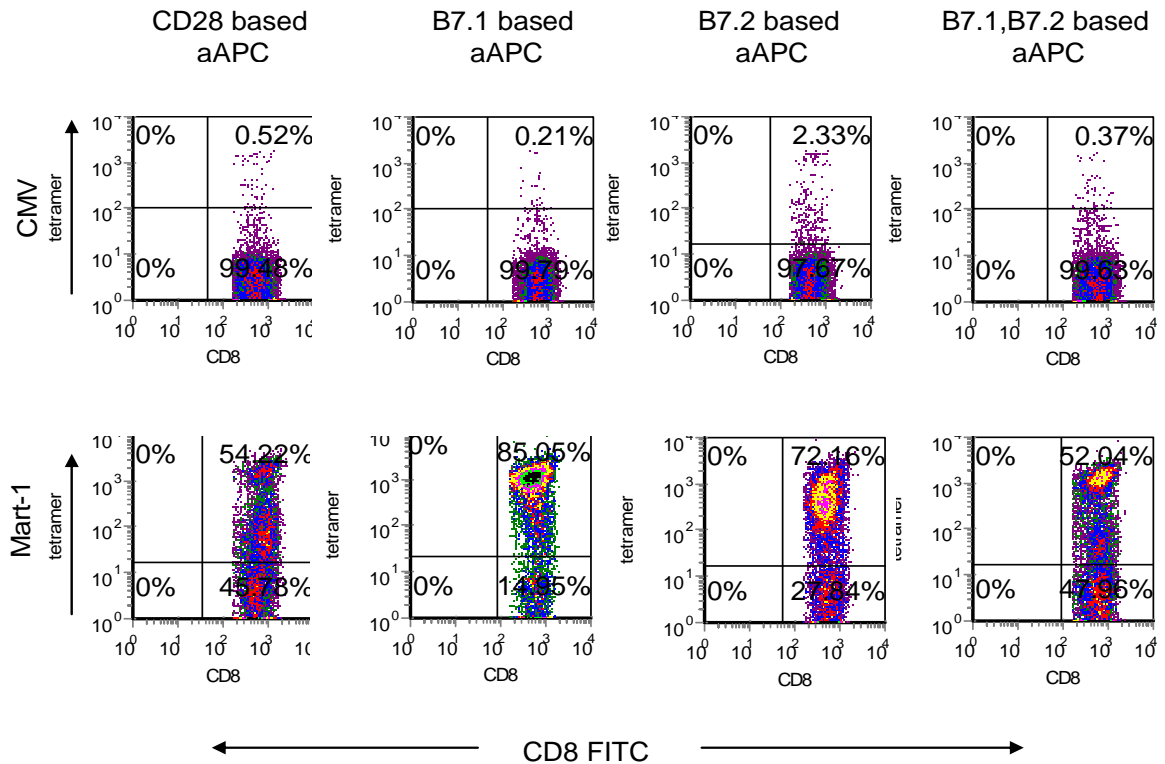
Legend: Figure 5 shows a tetramer analysis of Mart-1 specific CTL generated with 2nd generation B7-Ig based aAPC. The different aAPC formulations are indicated on top of each column. The ana;ysis shows that the B7.1-Ig based aAPC induced the highest frequency of antigen specific CTL. As negative control staining with the non-specific CMV tetramer was used.

Figure 6: Analysis of EphA2 specific CTL induced with either anti-CD28- or B7.1-Ig- based aAPC
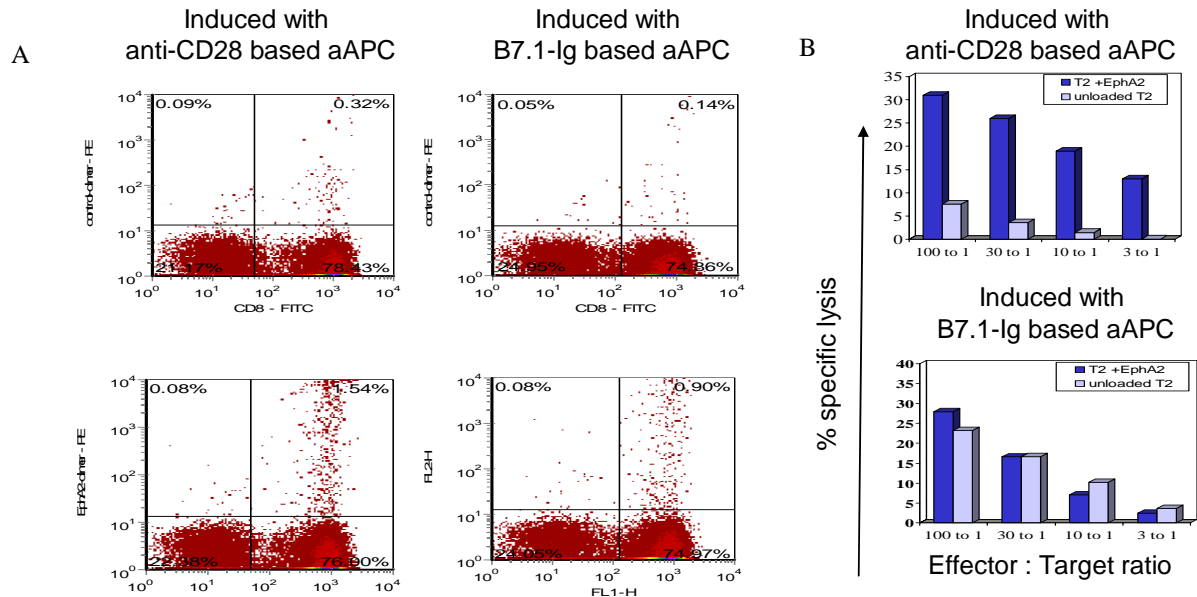

Legend: Figure 5 shows the analysis of the EphA2-specific CTL which were induced with B7.1-Ig based aAPC in comparison with EphA2-specific CTL induced with our current standard anti-CD28 based aAPC. In A is shown the tetramer analysis and in B is shown the result of the cytotoxic activity of the different EphA2-specific CTL lines determined with a standard ^{51}Cr-release assay. The effector target ratios are calculated according to the Total T cell number.

To evaluate the new CD83-Ig based aAPC we used the CMV system. We performed 3 experiments to generate CMV specific CTL from CD8+ T cells of healthy donors and compared the results with the use of our standard aAPC. The results show that the new aAPC are fully functional and capable of inducing antigen specific CTL.

Unfortunately, no major differences in specificity or expansion of the expanded T cells were detected when these new CD83-Ig based aAPC were compared with our current standard anti-CD28 based aAPC. Figure 7 shows one representative experiment. The fact that we were not able to major differences between the old and new aAPC could be due to the fact that the CMV system is such a strong antigen which does not require costimulation. Therefore, it is quite possible that we will detect differences when we will use these new aAPC to induce EphA2- specific CTL.

Figure 7: Tetramer analysis of 2nd generation aAPC induced CMV specific CTL
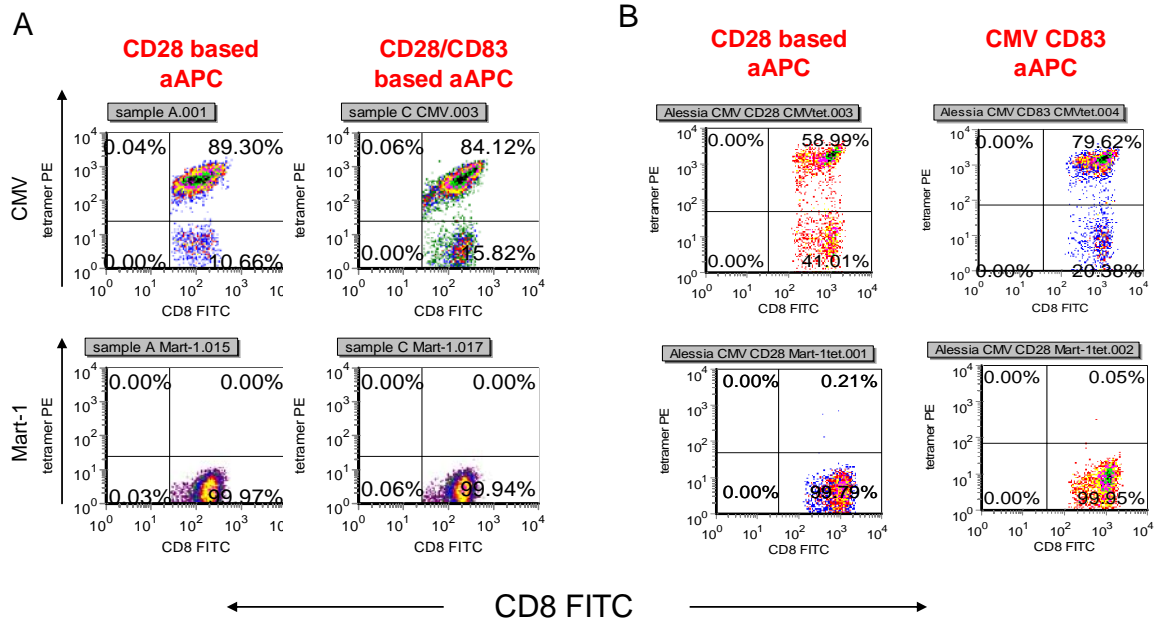

Legend: Figure 7 shows the dimer staining analysis of CMV specific CTL which were induced from CD8+ T cell of healthy donors using new aAPC made by coupling CD83-Ig +/- anti-CD28 together with HLA-A2-Ig onto magnetic beads. The result shows that all beads are functional, no major differences were seen between our standard anti-CD28 based aAPC and the new CD83-Ig based aAPC.

In addition to varying the type of costimulation we have also modified the ratio of signal 1 to signal 2 on our standard aAPC by preparing aAPC in the presence of different amounts of protein, as proposed in the statement of work. We have prepared a total of 4 different types of aAPC, by using the following ratio of HLA-A2-Ig to anti-CD28 (1:0, 8:2, 1:1, and 2:8). Figure 8 shows the results of our initial experiments, in which we used the different aAPC batches to generate CMV-specific CTL. While the CMV system is ideal to test the functionality of the new aAPC it seems it is to robust to identify differences in the stimulation capacity of the aAPC batches. Experiments to analyze the stimulation potential for EphA2-specific CTL are on going.

Finally, since the frequency of the prostate cancer specific CTL was quite low we used dimer and aAPC based antigen-specific T cell sorting to enrich for the antigen specific CTL and performed limiting dilution assays to clone EphA2 specific CTL. Currently we are expanding 30 potential T cell clones using a cloning protocol which we have previously successful established using influenza M1 specific CTL. The result of such cloning is shown in figure 9. Once the EphA2-specific clones are expanded to sufficient numbers we will test them and proceed with our *in vivo* experiments as proposed in the statement of work.

Figure 8: Tetramer analysis of CMV specific CTL after induction
with different aAPC
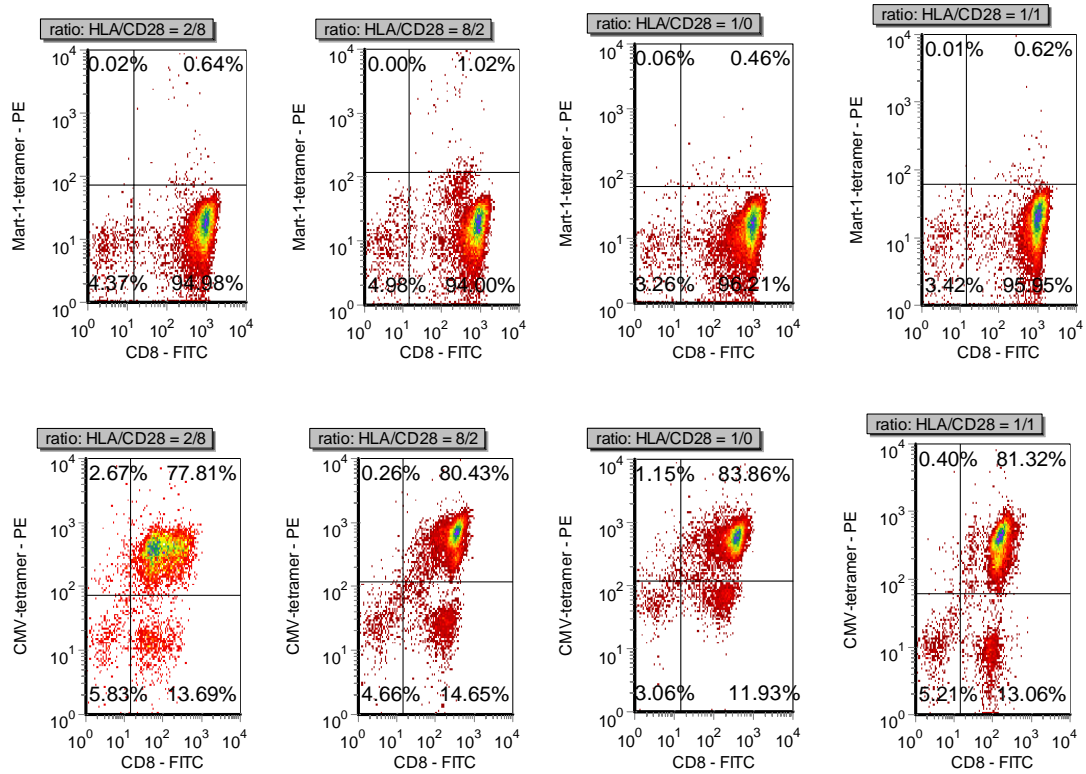
Legend: Figure 8 shows the CMV tetramer analysis of CMV specific CTL which were generated with aAPC which were made by coupling different amounts of HLA-A2-Ig and anti-CD28 onto a magnetic bead. The ration of signal 1 to signal 2 used for the aAPC preparation is shown in the title of each individual density plot. As negative control we stained the T cells with a PE labeled Mart-1 tetramer.

Figure 9: Tetramer analysis of an established flu-M1-specific T cell clone
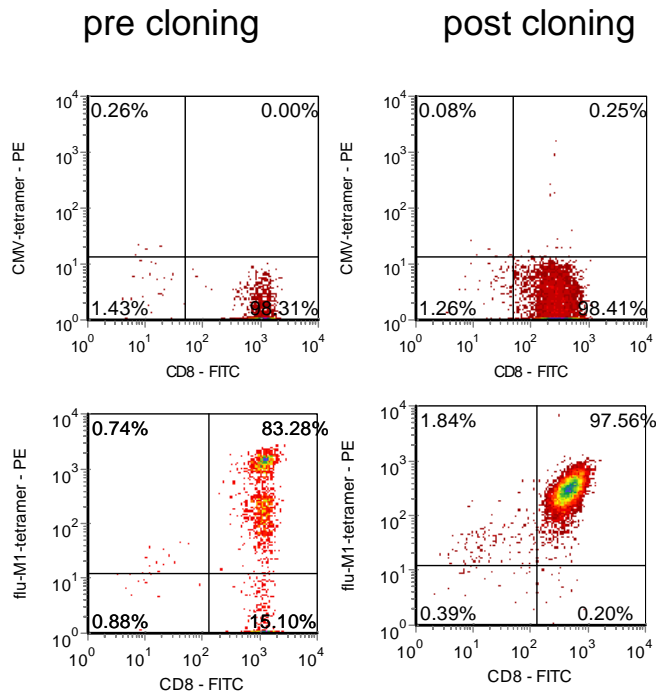
pre cloning
post cloning

KEY RESEARCH ACCOMPLISHMENTS

- aAPC can be used to induce functional active prostate cancer specific CTL.

- Development of 2nd generation aAPC using multiple costimulatory molecules and different ratios of HLA-Ig to the costimulatory molecule.

- While some variations from donor to donor and antigen to antigen were observed it seems that the engagement of CD28 with his natural ligand B7.1-Ig, on 2nd generation aAPC, instead of a mAb specific for CD28 can result in better stimulation and expansion of antigen-specific CTL. More detailed experiments are necessary to confirm initial results.

- aAPC as well as dimer technology can be used to enrich for antigen specific CTL.

REPORTABLE OUTCOMES

A manuscript describing the use of peptide loaded aAPC for generation and cloning of prostate cancer specific CTL is in preparation.

CONCLUSION

In summary, the performed experiments have resulted in the generation of prostate cancer specific CTL. We have further developed a large variety of 2nd generation aAPC which, while functional proven active; need to be further evaluated by using the low affinity prostate cancer specific antigens EphA2 and PSMA. These results will permit us to move effectively and clearly into evaluation of the in vivo efficacy of aAPC expanded prostate cancer specific T cells. In addition we are currently cloning the aAPC induced and enriched EphA2 specific CTL which will then be used for further *in vivo* experiments as proposed in the statement of work as well as to explore the potential of the 2nd generation aAPC to further expand these T cell clones in an antigen specific manner.

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